

Reprinted from the *American Journal of Physiology*.

VOL. XXVI. — OCTOBER 1, 1910. — No. VII.

THE PREPARATION AND PROPERTIES OF
THROMBRIN, TOGETHER WITH OBSER-
VATIONS ON ANTITHROMBIN AND
PROTHROMBIN.

By W. H. HOWELL.

[FROM THE PHYSIOLOGICAL LABORATORY OF THE JOHNS HOPKINS UNIVERSITY.]

C.

WELLCOME INSTITUTE LIBRARY	
Coll.	we/VOrnec
Coll.	pam
No.	WH 310
	1 7 10
	H 85 p



22500883121

THE PREPARATION AND PROPERTIES OF THROMBIN, TOGETHER WITH OBSERVATIONS ON ANTITHROMBIN AND PROTHROMBIN.

By W. H. HOWELL.

[*From the Physiological Laboratory of the Johns Hopkins University.*]

IN a paper from this laboratory by Rettger¹ some facts were given which throw doubt upon the usual view that thrombin is an enzyme or ferment. According to these experiments thrombin in solution is not easily destroyed by boiling, and in its reaction with fibrinogen it does not appear to act as a catalytic or pseudo-catalytic agent. In order to carry these experiments farther it seemed necessary to devise a more satisfactory method of preparing thrombin, and the present paper constitutes a preliminary report upon a new method of isolating this substance. As is well known, the two methods usually employed in physiology to prepare thrombin are, the method of Schmidt, according to which serum is precipitated by excess of alcohol and the precipitate after standing some time under alcohol is dried and extracted with water; and the method of Buchanan as modified by Gamgee² and afterward by Lea and Green,³ which consists in extracting washed fibrin with 8 per cent solution of sodium chloride. Both of these methods, but especially the latter, give solutions which show a strong thrombin action. The solutions obtained by these methods contain much protein in addition to the thrombin. The solution obtained by Schmidt's method contains protein which in the extract as prepared does not coagulate upon boiling. If, however, neutral salts are added to the solution in slight concentration, particularly ammonia salts or salts of the alkaline earths, a heavy precipitate is produced on heating the solution. If the method of extracting the

¹ RETTGER: This journal, 1909, xxiv, p. 406.

² GAMGEE: Journal of physiology, 1879, ii, p. 145.

³ LEA and GREEN: *Ibid.*, 1884, iv, p. 380.

washed fibrin with strong saline is used, the solution contains much protein. When fresh fibrin from the dog is used, and, after thorough washing in water, is digested for twenty-four hours with 8 per cent solution of sodium chloride at 40° C., it is nearly all dissolved, and the filtered solution gives a very abundant precipitate on heating to 57° to 60° C., and usually a second much smaller precipitate when heated further to 76°–80° C. The presence of these large amounts of protein in solution makes it difficult or impossible to study satisfactorily the properties of the thrombin itself. An effort was made accordingly to remove the protein without precipitating the thrombin. A method was finally devised which effected this result, as follows:

METHOD OF PREPARING PURE THROMBIN.

A large quantity of fibrin obtained by whipping pig's blood is thoroughly washed in running water until it is entirely freed from hæmoglobin. The process is tedious, taking several hours, as the fibrin must be continually kneaded in the running water, and the larger masses must be shredded by hand to remove the enclosed hæmoglobin. The white mass finally obtained is minced finely and thoroughly squeezed in cheese cloth, and is then placed in a vessel and covered with an 8 per cent solution of sodium chloride. This preparation is kept in the ice chest for forty-eight to seventy-two hours and is then filtered, first through cheese cloth and then through filter paper. The somewhat viscid solution thus obtained has a powerful thrombin action. To remove the coagulable protein contained in it it was shaken repeatedly with chloroform, adding each time about one half its volume of chloroform, shaking vigorously in the hand or a shaking machine and then filtering through filter paper. At first a heavy precipitate is produced and the liquid above the chloroform is turbid and gives a turbid filtrate. But as the process is repeated again and again, the filtrate becomes clearer and comes through more rapidly. Each time that this filtrate is shaken with new chloroform, the latter quickly forms an emulsion which on standing sinks to the bottom. When the filtrate is entirely clear, or shows only a slight opalescence when seen in bulk, it continues to form an emulsion when shaken with the chloroform, and the process must be continued until after prolonged shaking with fresh chloroform the latter on standing forms a clear layer without

evidence of an emulsion. The water-clear filtrate is then examined by heating a specimen to boiling in a test tube. If a turbidity appears, the process of shaking with chloroform must be continued one or more times until a specimen on heating gives no opalescence whatever, even after the addition of some crystals of ammonium sulphate. The clear liquid thus obtained free from all protein coagulable by heat is still rich in thrombin. A few drops added to a solution of pure fibrinogen will cause a firm clot within one or two minutes, the liquid usually becoming first markedly opalescent and then setting to a firm jelly. The process of purifying the thrombin is time-consuming, requiring a number of days, and is attended by a considerable loss of liquid. At each filtration the emulsion of chloroform holds back on the filter some of the solution. The chloroform itself, towards the end at least, when the protein is small in amount, can be preserved and redistilled. If the process is carried out as rapidly as possible, the solution finally obtained contains, as stated above, much thrombin and none of the protein originally present which is coagulable by heat in the presence of neutral salts. This solution, if allowed to stand saturated with chloroform, will, in the course of weeks, lose its coagulating effect on fibrinogen solutions. To keep the thrombin permanently it must be evaporated to dryness as rapidly as possible at a low temperature, 35° to 40° C. My method of doing this has been to place 5 to 10 c.c. of the solution in watch crystals and allow the evaporation to occur during ten or twelve hours in a warm chamber kept at 35° to 40° C. The white residue obtained is mixed with crystals of sodium chloride, and is quickly and completely dissolved on the addition of water. If larger amounts are evaporated and the time of evaporation is thereby much prolonged, the residue may be slightly or deeply colored in yellow and on addition of water a portion of it is found to be insoluble. Corresponding to this change its coagulating effect upon solutions of fibrinogen is diminished or destroyed altogether, so that evidently prolonged exposure to 40° C. in solution brings about a slow decomposition of the thrombin, converting it to a substance insoluble in water. When evaporated in small quantities in watch crystals as described above, the preparations of thrombin are apparently entirely stable. Months afterward the residue dissolved in water shows a prompt and powerful action upon solutions of fibrinogen. So far as my experience goes, such specimens of thrombin may be kept indefinitely. To ob-

tain the thrombin free from the sodium chloride, the solution, before being evaporated, may be dialyzed through collodion tubes. As is well known, crystalloids pass through a collodion membrane very rapidly, so that after a dialysis of five or six hours against a large amount of distilled water, renewed once or twice, the thrombin solution is freed from the sodium chloride, and may then be evaporated to dryness in watch crystals at a low temperature as described. I have been successful in obtaining a number of specimens of thrombin in this way free from sodium chloride. The residue is slight in amount, has a crystalline structure under the microscope, is readily and quickly dissolved in water, and has a powerful coagulating effect upon fibrinogen. Whether or not the crystalline structure shown by these preparations is due to the thrombin or to the presence of minute amounts of inorganic salts was difficult to determine. The crystals had a snowflake appearance, and as far as could be determined they were not entirely destroyed by heating the preparations to the point of carbonization. It seems most probable that the crystals seen in the preparations consisted of minute amounts of salts of some sort not dialyzed off, and that these overlay a granular material also evident which was the thrombin. In preparing my specimens of thrombin I subsequently gave up the process of dialyzing for two reasons. In the first place, it happens in some cases that much of the thrombin disappears during the dialysis, presumably because it is able to diffuse slowly through the collodion membrane. While it is possible to choose such a time for dialysis as will remove the sodium chloride and still leave much thrombin, there is always the possibility that the dialysis may be prolonged so far as to lose most of the thrombin. In the second place, the solutions of thrombin obtained free from sodium chloride by dialysis are somewhat more unstable than the saline solutions when exposed to heat, and are therefore more liable to be decomposed during the process of evaporation. As the presence of the sodium chloride does not interfere with the study of the properties and reactions of the thrombin and seems to confer a greater stability upon its solutions, it was found preferable in the later preparations to evaporate the solutions of thrombin to dryness without previous dialysis.

REACTIONS OF THE THROMBIN.

Solutions of the thrombin before and after dialysis were tested repeatedly by the usual protein reactions and in all cases with positive results. The following reactions were noted:

1. A solution of thrombin remains entirely clear upon boiling even in the presence of salts, including ammonium sulphate (2 to 5 per cent). The thrombin is therefore not coagulated by heat in neutral solutions.

2. It is imperfectly precipitated by a large excess of alcohol.

3. It is precipitated by half saturation with ammonium sulphate, and the precipitate even after standing for several days is readily soluble in water and shows its usual action in coagulating a solution of fibrinogen.

4. It gives a positive biuret reaction.

5. It gives a very distinct tryptophan reaction by the method of Adamkiewicz, using a freshly prepared mixture of sulphuric and acetic acids.

6. It gives a positive xanthoproteic reaction.

7. It gives a positive reaction with Millon's reagent.

8. The reaction for sulphur is negative or very feeble.

9. The reaction with Molisch's reagent is negative.

10. The reaction for phosphorus is negative.

11. With acetic acid and potassium ferrocyanide it gives a faint precipitate; with potassium ferrocyanide alone in neutral reaction no precipitate or turbidity; hence probably it is not a basic protein.

12. Careful addition of dilute ammonia in the presence of ammonia salts gives no precipitate; hence it is not a protein of the histone group.

13. Nitric acid in excess gives a faint turbidity which clears on heating and reappears on cooling. According to these reactions the thrombin must be classed as a simple protein which does not however fall into any of the groups usually described.

14. While the solutions of thrombin prepared as described do not give an opalescence or precipitate when boiled in the presence of neutral salts, it was found that if given an acid reaction with acetic acid boiling causes a turbidity or precipitate unless the solution is very dilute. In the latter case the solution while clear when heated became turbid on cooling, the turbidity disappearing upon heating and returning upon cooling. This reaction naturally gave rise to the sus-

picion that my solutions still contained some trace of the usual blood proteins. Accordingly, I again submitted it to repeated shaking with chloroform, testing each filtrate by boiling a specimen after acidifying with acetic acid. While shaking with the chloroform no longer gave any appearance of an emulsion, it was noticed that after each treatment there was a slight membranous layer upon the surface of the chloroform. By continuing this treatment a filtrate was finally obtained which on boiling after acidifying with acetic acid gave no turbidity upon cooling. Upon testing this filtrate upon solutions of fibrinogen, it was found that the thrombin had entirely disappeared, although the original solution had shown a very strong thrombin action. When tested for protein by means of the biuret, Millon, and tryptophan reactions, the solution gave evidence of the presence of very minute amounts of protein, scarcely detectible. The conclusion to be drawn from this result is that the protein which gave the reaction with acetic acid was not an impurity but the thrombin itself, and that among its distinctive reactions we must include this one, namely, that while incoagulable on heating in neutral solutions it gives in dilute solutions, after acidifying with acetic acid and boiling, a solution which remains clear when hot and becomes turbid on cooling.

15. As stated above, thrombin solutions when saturated with chloroform very gradually lose their activity after a period of weeks or months, depending on the temperature. Aqueous solutions without chloroform or other antiseptic, such as toluol, soon undergo putrefaction, and it is noteworthy, as pointed out by Rettger, that this process at first serves to increase the activity of the thrombin in its coagulating effect on pure fibrinogen. Specimens of washed fibrin suspended in water show a marked thrombin action after putrefaction is well advanced, and this action is maintained for a long period but eventually disappears. It is more striking, perhaps, that specimens of pure thrombin in aqueous solution on undergoing putrefaction, show an increased efficiency as judged by the rapidity with which they coagulate fibrinogen solutions, although eventually, if allowed to stand, the thrombin disappears. This fact suggests that the action of putrefaction on the molecule of thrombin splits off a complex which acts more rapidly on the fibrinogen than the original thrombin. There is some reason to believe that this efficient complex is characterized by the presence of the indol grouping, but the experiments to deter-

mine this point have not yet been brought to a satisfactory termination.

16. *Effect of temperature on the thrombin.*—As stated above, aqueous solutions of thrombin protected from putrefaction slowly lose their efficiency, and this gradual alteration in the thrombin is accelerated by a high temperature. Rettger has shown that aqueous solutions of thrombin prepared by Schmidt's method are weakened but not totally destroyed by short exposures to the temperature of boiling. I have repeated these experiments upon my specimens of pure thrombin and find that those specimens which contain some sodium chloride may be boiled for a few minutes without destroying their activity, although the activity is diminished, as shown by the longer time required to coagulate a given specimen of fibrinogen. On the contrary, solutions of thrombin which have been dialyzed previously to remove the sodium chloride may lose their coagulating effect on fibrinogen completely after boiling for a minute or two. This fact coincides with the result previously mentioned, that dialyzed solutions of thrombin are more liable to alteration than the undialyzed solutions in slow evaporation at a temperature of 35° – 40° C. The presence of the sodium chloride makes the thrombin more resistant to the effects of high temperature. Such experiments as the following seem to demonstrate this point: Three specimens of a dialyzed solution of thrombin were taken, 2 c.c. each; one was diluted with an equal amount of water, one with an equal amount of a 2 per cent solution of sodium chloride, and one with an equal amount of an 8 per cent solution of sodium chloride. Each specimen was brought to a boiling temperature for a minute, and then 13 drops of each were added to 4 c.c. of a fibrinogen solution. The specimens were left over night. Those with sodium chloride, 1 and 4 per cent, gave excellent firm clots; the one without sodium chloride gave only an extremely feeble coagulum. The short exposure to boiling had almost but not completely destroyed the thrombin contained in it.

THE QUANTITATIVE RELATIONSHIP OF THROMBIN TO FIBRINOGEN.

The most important factor for consideration in discussing the question of whether or not thrombin acts as an enzyme or catalytic agent is the quantitative relationship between the thrombin and the fibrin-

ogen converted to fibrin. According to the usual belief thrombin acts as an enzyme, and Schmidt has given some experiments intended to show that the thrombin is not used up in the action which it causes, but may be active over and over again after the manner of a catalytic reagent. An examination of Schmidt's experiments will show, as I have pointed out elsewhere,⁴ that they are open to the obvious objection that he began his experiments with a large excess of thrombin. To 10 c.c. of a "salt-plasma solution" he added 80 c.c. of a thrombin solution. The seven successive coagulations which he obtained by using the serum of each clot to coagulate a new specimen of salt plasma may have been due therefore to the excessive amount of thrombin with which he started rather than to the fact that the same thrombin acted upon seven successive lots of plasma. His seventh coagulation required four hours, while his first occurred within two minutes. Rettger,⁵ on the contrary, describes an experiment in which the amount of fibrin obtained from equal volumes of a solution of fibrinogen was found to vary in proportion to the amount of thrombin added, when the amount of this latter substance was kept below the quantity necessary to coagulate the whole of the fibrinogen. A result of this kind indicates a definite quantitative relation between the amount of thrombin and the amount of fibrin formed such as would not be expected if the thrombin plays the part of an enzyme. In the present investigation the opportunity seemed to be offered to test this point with more care, making use of known amounts of fibrinogen and thrombin. The object of the experiments was twofold, — in the first place to determine whether, when submaximal amounts of thrombin are used, the amount of fibrin formed varies with the amount of thrombin added, and, in the second place, making use of small amounts of thrombin, to see if the amount of fibrin formed varies with the time during which the thrombin acts. The experiments proved to be time-consuming, and only four were completed. One of these was useless owing to the fact that too much thrombin was used. Two of the experiments were successful and gave results which, while positive as regards the questions stated above, brought to light a new and interesting difficulty. The details of these experiments are as follows:

Preparation of the thrombin. — The thrombin used had been pre-

⁴ HOWELL: The coagulation of blood, Cleveland medical journal, January and February, 1910.

⁵ RETTGER: *Loc. cit.*

pared by the chloroform method described above. The solution had been evaporated to dryness in watch crystals without previous dialysis. The dry preparations contained, therefore, a quantity of sodium chloride. To estimate the amount of thrombin the following method was used: The contents of each crystal were dissolved in 10 c.c. of water, giving a solution that contained approximately 2 per cent of sodium chloride; 5 c.c. of this solution were evaporated to dryness on the water bath in a weighed platinum crucible, and the crucible with its residue was then heated to 105° C. in an electric oven until the weight was constant. The weight of the total residue, sodium chloride and thrombin, was thus obtained. The crucible was then heated over a small flame until the organic material was incinerated and was again weighed. The difference between the two last weighings gave the weight of the thrombin in 5 c.c. of the solution used. This method did not allow for the weight of any ash that the thrombin itself gives, but the total amount of thrombin used in the experiments, namely, from 0.05 to 0.64 mgm., was so small that this error is negligible. In the first two experiments each cubic centimetre of the solution contained 3.2 mgm. of thrombin, in the third each cubic centimetre contained 2.5 mgm. of thrombin. For the coagulation experiments with fibrinogen 1 c.c. of these solutions was diluted to 10 c.c. with water, and from 2 c.c. to 0.2 c.c. of this diluted solution was used, containing from 0.64 to 0.05 mgm. of thrombin.

Preparation of the fibrinogen. — The fibrinogen used was prepared in all cases from clear plasma obtained from cats which had fasted for twenty-four hours. The method used was a modification of that given by Hammarsten and has been described in previous papers from this laboratory. The plasma obtained by centrifugalizing the oxalated cat's blood was precipitated by the addition of an equal volume of saturated solution of sodium chloride. This mixture was centrifugalized and the supernatant liquid poured off from the precipitate. It is an advantage to centrifugalize the mixture as soon as it is made, otherwise the fibrinogen collects in large flocculi which may rise to the top of the tube during centrifugalization and give difficulty in decanting. The precipitate of fibrinogen in each tube was washed with a half-saturated solution of sodium chloride and then dissolved in a 2 per cent solution of sodium chloride, filtered and again precipitated by addition of an equal volume of saturated solution of sodium

chloride. This precipitate was centrifugalized, washed, dissolved in 2 per cent solution of sodium chloride, filtered and again precipitated by half saturation with sodium chloride. After the third precipitation the washed precipitate was finally dissolved in a 0.9 per cent solution of sodium chloride. Sometimes, in fact as a rule, the third precipitate failed to dissolve in the dilute sodium chloride, but in such cases the addition of a drop of a 5 per cent solution of sodium carbonate was sufficient to carry the fibrinogen into solution. This final solution was filtered and was then dialyzed in collodium tubes for about twelve hours against a large volume of a 0.9 per cent solution of sodium chloride, the outside solution being changed once. Solutions of fibrinogen made in this way do not, as a rule, undergo spontaneous coagulation, no matter how long they are kept, although after a certain time they may precipitate. Rettger found it advisable before dialyzing the final solution to treat it with dilute sodium phosphate and barium chloride on the view that the delicate precipitate of barium phosphate in settling out will carry down any remnant of thrombin or prothrombin present in the solution. If care is exercised in the preparations, this additional procedure does not seem to be necessary.

In the experiments made with these preparations a known amount of thrombin was added to given volumes of the fibrinogen solution, and, after standing for definite times, the clot of fibrin formed was twisted out upon a glass rod, placed on a weighed filter paper, washed successively with solutions of sodium chloride 0.9 per cent, cold water, and hot water until the washings were free from chlorides. The fibrin was washed finally with alcohol and ether, dried and then heated in an electric oven at 105° C. to constant weight.

Experiment I. Plasma from 3 cats, fibrinogen prepared and after dialysis two specimens of 35 c.c. each taken for coagulation. To one was added 2 c.c. of the dilute thrombin solution (0.64 mgm. thrombin), to the other 0.5 c.c. (0.16 mgm. thrombin). The mixtures were allowed to stand for twenty-four hours. Solid clots were formed, that with the more thrombin (I) looking much more dense. Clots twisted out with a glass rod, placed on weighed filters and washed and heated as described above.

I.	35 c.c. fibrinogen solution	+ 0.64	mgm. thrombin
	Weight of tube + filter	=	11.4118
	Weight of tube + filter + fibrin	=	<u>11.4543</u>
	Weight of fibrin	=	.0425

II. 35 c.c. fibrinogen	+ 0.16	mgm. thrombin
Weight of tube + filter	=	13.0130
Weight of tube + filter + fibrin	=	13.0470
Weight of fibrin	=	.0340

That is to say, 0.16 mgm. of thrombin gave 34 mgm. of fibrin, while 0.64 mgm. of thrombin gave 42.5 mgm. of fibrin.

Experiment II. Plasma from two cats, somewhat milky from fat. Fibrinogen prepared and after dialysis two specimens of 25 c.c. each taken. To each of these there was added 0.5 c.c. of the dilute thrombin solution (0.16 mgm. thrombin). One specimen was allowed to stand twenty-four hours, the other forty-eight hours.

I. 25 c.c. fibrinogen solution + 0.16 mgm. thrombin, twenty-four hours.

Weight of tube + filter	=	12.4878
Weight of tube + filter + fibrin	=	12.5045
Weight of fibrin	=	.0167

II. 25 c.c. fibrinogen solution + 0.16 mgm. thrombin, forty-eight hours.

Weight of tube + filter	=	16.0442
Weight of tube + filter + fibrin	=	16.0612
Weight of fibrin	=	.0170

The difference between the two yields of fibrin was only 0.3 mgm. It would seem, however, that in this experiment the fibrinogen solution was much more dilute than in the first experiment, and probably the amount of thrombin added was too large, that is to say, too near the amount necessary to give a maximal formation of fibrin. The experiment is of value, not so much in determining the influence of time upon the amount of fibrin formed with sub-maximal doses of thrombin as in giving an indication of the degree of accuracy in the method used for determining the fibrin.

Experiment III. Plasma from four cats, obtained 200 c.c. of clear plasma, fibrinogen prepared as usual. After dialysis took 3 specimens of 50 c.c. each. Added to one 0.2 c.c. of diluted thrombin (0.05 mgm.) and kept twenty-four hours; to another the same amount of thrombin and kept seventy hours; and to the third 1 c.c. of diluted thrombin solution (0.25 mgm.) and kept twenty-four hours. The last specimen was clotted in two hours, while the first two began to show the initial opalescence

at the fourth hour. At the end of twenty-four hours all had reached a final stage, and it was evident to the eye that the clot in the third specimen was much heavier than in the other two.

- I. 50 c.c. fibrinogen solution + 0.05 mgm. thrombin, stood twenty-four hours.

$$\begin{array}{rcl} \text{Weight of tube + filter} & = & 12.0631 \\ \text{Weight of tube + filter + fibrin} & = & 12.0744 \\ \hline \text{Weight of fibrin} & = & .0113 \end{array}$$

- II. 50 c.c. fibrinogen solution + 0.05 mgm. thrombin, stood seventy hours.

$$\begin{array}{rcl} \text{Weight of tube + filter} & = & 15.9820 \\ \text{Weight of tube + filter + fibrin} & = & 15.9922 \\ \hline \text{Weight of fibrin} & = & .0102 \end{array}$$

- III. 50 c.c. fibrinogen solution + 0.25 mgm. of thrombin, stood twenty-four hours.

$$\begin{array}{rcl} \text{Weight of tube + filter} & = & 15.5892 \\ \text{Weight of tube + filter + fibrin} & = & 15.6260 \\ \hline \text{Weight of fibrin} & = & .0368 \end{array}$$

So far as this experiment goes, therefore, it would appear that the larger the amount of thrombin added the greater is the amount of fibrin formed, provided the amount of thrombin is kept below that necessary to convert all of the fibrinogen to fibrin—moreover, with a minimal amount of thrombin the amount of fibrin formed is not increased by longer standing. Both of these facts are opposed to the theory that the thrombin acts after the manner of an enzyme. If the data from the first and the third experiment are compared, they give us the following series:

0.05	mgm.	thrombin	yields	10.75	mgm.	fibrin,	or	1	to	215
0.16	"	"	"	34.00	"	"	or	1	to	212.5
0.25	"	"	"	36.80	"	"	or	1	to	147
0.64	"	"	"	42.50	"	"	or	1	to	66

According to these results the amount of fibrin formed when minimal amounts of thrombin are used is directly proportional to the weight of thrombin, or, in other words, a definite amount of thrombin converts a definite amount of fibrinogen to fibrin. One part of throm-

bin can combine with or react with 212 to 215 parts of fibrinogen at a maximum. When the thrombin is increased beyond a certain amount, this proportionality disappears. As the thrombin is increased, the amount of fibrin formed is increased but not in direct proportion. Some of the thrombin is apparently inactive, possibly because it is physically adsorbed by the fibrin formed. In order to convert all of the fibrinogen to fibrin it is necessary to add thrombin in much larger amounts than is indicated by the ratio of 1 to 215.

The conclusions to be drawn from the experiments given above are, however, much complicated by the following facts: When the fibrin formed in one of these specimens is twisted out upon a glass rod and the remaining liquid is allowed to stand for a number of hours, a new lot of fibrin forms. If this is removed in time, a third lot of fibrin appears in the course of twenty-four hours. In Experiment III, for example, specimens 1 and 2 gave four successive clots, while specimen 3 gave three successive clots. Thus the original clot in 1 was removed at the end of twenty-four hours. At the end of forty-eight hours a new clot equally voluminous had formed, and when this was removed a third clot of about the same amount had formed at the end of seventy-two hours. Meanwhile specimen 2, exactly similar to 1, which had been standing for seventy hours without being disturbed, had only as much fibrin as in the first clot of specimen 1. Evidently, after a certain amount of fibrin is formed with a given submaximal quantity of thrombin, an equilibrium is reached due to some influence of the fibrin itself, since the removal of this fibrin at once permits the conversion of a new quantity of fibrinogen to fibrin. In specimens 1 and 2, containing each 0.05 mgm. thrombin, four successive and approximately equal clots were formed. After the last clot was removed the liquid gave no further fibrin, but when heated to 60° C. there was an abundant precipitate, showing that the fibrinogen had not all been converted to fibrin. In specimen 3, containing five times as much thrombin (0.25 mgm.), three successive clots were obtained. After the removal of the last one no further clotting occurred, and the liquid when heated to 60° gave only a minute opalescence. Evidently all of the fibrinogen had been converted to fibrin.

A satisfactory explanation or discussion of this phenomenon is not possible without additional experiments to answer the numerous questions that suggest themselves. As in the case of enzymes, the reaction

caused by the thrombin is incomplete owing to the fact that the end product of the reaction, the fibrin, inhibits in some way the further action of the thrombin, and this condition of equilibrium is disturbed by the removal of the fibrin. On the other hand, the reaction differs from that of enzymes in two respects. In the first place, in the fact that the portion of the fibrinogen converted to fibrin increases as the amount of thrombin is increased, although not proportionally. If sufficient thrombin is added, all of the fibrinogen is changed to fibrin. In the second place, when a submaximal amount of thrombin is used, the portion of fibrinogen converted to fibrin does not vary with the time that the thrombin is allowed to act. Why the mechanical removal of the fibrin should again start up the thrombin action is difficult to understand. The simplest suggestion, perhaps, is that the fibrinogen combines with a definite quantity of thrombin, or, to be more cautious, a certain amount of thrombin reacts with a certain quantity of fibrinogen, but the fibrin thus formed is able to hold in loose union by mechanical adsorption an additional amount of thrombin. It is this loosely combined thrombin which is liberated by the mechanical process of twisting out and squeezing the fibrin, and is able when set free to precipitate a new lot of the fibrinogen. This suggestion carries with it the conclusion that the maximum ratio of combination by weight of the fibrinogen and thrombin given above, namely, 215 to 1, does not represent a real maximum, since the fibrin held in loose combination a portion of the thrombin which had not reacted with fibrinogen. The maximum ratio of combination of the thrombin would be obtained by determining the total weight of fibrin formed in the successive coagulations. This explanation is not insisted upon, as further experiments may fail to support it. It may be added that the author has observed previously a similar reaction in horse's plasma. If after the plasma is coagulated the clot is broken up and filtered off, the filtrate may clot again, and the process may be repeated a number of times. A somewhat similar process has also been described by the author in connection with the heat coagulation of the proteins of the serum of limulus. When the coagulum obtained at a given temperature is filtered off and the clear filtrate is again heated to the same temperature, a new coagulation occurs, and this process may be repeated several times.

THE ANTITHROMBIN OF PEPTONE PLASMA.

A number of experiments were made with peptone plasma from the dog to determine whether it contains a substance capable of neutralizing the action of thrombin on fibrinogen. The peptone plasma was prepared by injecting into the femoral artery of a fasting dog a 6 to 7 per cent solution of peptone in quantity to yield 0.3 to 0.4 gm. of peptone to each kilogram of animal. Twenty minutes after this injection the animal was bled from the carotid, and the specimen of blood removed was centrifugalized to obtain a clear plasma. In successful cases the plasma remained unclotted for at least forty-eight hours. For the sake of comparison, a specimen of the dog's blood was removed before the injection of the peptone, and was received into an oxalate solution and centrifugalized. The two plasmas from the same animal thus obtained will be spoken of briefly as oxalated plasma and peptone plasma. It was found that the oxalated plasma clotted firmly in a few minutes on the addition of a few drops of thrombin solution; the peptone plasma, on the contrary, as has been observed by others, was not affected by the addition of amounts of thrombin which for the same amount of normal plasma would have meant a large excess. The most decisive experiments were made with fibrinogen solutions to which were added certain amounts of peptone plasma and thrombin. Similar tests were made for the sake of control with mixtures of fibrinogen solutions with thrombin alone or with thrombin and oxalated plasma. These experiments brought out very clearly the positive fact that peptone plasma contains something which prevents thrombin from acting on fibrinogen. The following typical experiment may be quoted:

Fibrinogen solution	2.5 c.c.	} This mixture clotted firmly in two minutes.
Sodium chloride solution 0.9 per cent	2 c.c.	
Thrombin solution (2.5 mgm.) . .	1 c.c.	
Fibrinogen solution	2.5 c.c.	} This mixture clotted firmly within two minutes.
Oxalated plasma	2 c.c.	
Thrombin solution (2.5 mgm.) . .	1 c.c.	
Fibrinogen solution	2.5 c.c.	} This mixture showed no sign of clotting after twenty-four hours.
Peptone plasma	2 c.c.	
Thrombin solution (2.5 mgm.) . .	1 c.c.	

Results of this kind were obtained uniformly and furnish convincing proof that in the peptone blood something is contained which not only prevents the coagulation of that plasma, but is capable of antagonizing thrombin added to it in considerable amounts, or finally prevents thrombin from acting on a solution of fibrinogen. The substance contained in the peptone plasma which prevents its clotting exerts this action no doubt by antagonizing the effect of thrombin. This substance is designated usually as antithrombin, but whether it acts by combining directly with the thrombin has not been demonstrated, although it is made probable by the experiments cited above. That it is an organic substance present in the plasma is indicated by the effect of heating. Peptone plasma heated for ten minutes at 60° and filtered from the heat coagulum of fibrinogen formed at this temperature, still prevents entirely the action of thrombin on fibrinogen, whereas when the plasma is heated to 75° – 80° C. and is filtered from the larger heat coagulum it loses completely its power of antagonizing the action of thrombin on fibrinogen. We may say, therefore, that the antistubstance present in peptone plasma is not affected by heating for ten minutes at 60° C., but is destroyed when the temperature rises as high as 75° to 80° C.

An interesting feature of the peptone plasma is the effect of diluting it with water and with physiological saline respectively. In a previous paper the author has called attention to the fact that terrapin's plasma, obtained in the fall when the animal is in good condition preparatory to the winter hibernation, does not clot spontaneously. When diluted with water, it clots readily, while diluting with sodium chloride solution 0.9 per cent has no such effect. This fact was used as an argument against the view that the incoagulability of terrapin and bird blood is due to the presence of an antithrombin. My present experience with peptone plasma shows, however, that this argument was not justified. Peptone plasma seems undoubtedly to contain an antistubstance to the thrombin, yet when diluted with water it may be made to clot, while an equal dilution with 0.9 per cent solution of sodium chloride has no such effect. The amount of dilution with water necessary to cause clotting in the peptone plasma varied in my experience in the different specimens of blood. The difference depends, no doubt, on the amount of antistubstance present in the plasma. With specimens of peptone plasma containing an amount of antistubstance sufficient to

prevent the plasma from clotting for at least forty-eight hours, dilution with five times its volume of water caused clotting after a few hours, while an equal dilution with saline solution remained unclotted for over twenty-four hours at least. If this reaction in peptone plasma is associated with the presence of an antithrombin, then we may infer that the normal terrapin's (and bird's) plasma which shows a similar reaction may likewise owe its non-coagulability to the presence of an antistubstance. It may be added that dilution of oxalated plasma with water does not cause coagulation.

Experiments upon prothrombin. — It seemed probable that the chloroform method of isolating thrombin might be used successfully to isolate its antecedent substance, the so-called prothrombin. For this purpose the blood of a fasting cat was received into oxalate solution, and, after centrifugalizing, the clear plasma was dialyzed against 0.9 per cent salt solution for twenty-four hours to get rid of the excess of oxalate. The dialyzed plasma was then shaken with chloroform for one to two hours and filtered. The filtrate clotted. This result showed that the method is not applicable to the isolation of prothrombin, but it is interesting as proving that prothrombin can be converted to thrombin in a calcium-free solution. Whatever may be the mode of action of calcium salts in activating prothrombin to thrombin, it is evident that other conditions may effect the same activation, although in a less effective manner.

A further experiment was made upon prothrombin to determine in how far it is precipitated from oxalated plasma in the preparation of fibrinogen. Mellanby has assumed an intimate relationship between the fibrinogen and prothrombin in normal plasma which it seemed possible to test by a simple experiment. For this purpose clear oxalated plasma was prepared from cat's blood. From this plasma the fibrinogen was precipitated by the addition of sodium chloride until the liquid was three quarters saturated. The precipitated fibrinogen was centrifugalized off, was washed twice with half-saturated solution of sodium chloride, and was then dissolved in a 2 per cent solution of sodium chloride. It was found that this solution did not clot spontaneously after twenty-four hours, that it did not clot upon the addition of an equal volume of 0.9 per cent solution of sodium chloride, but that it did clot quite promptly upon the addition of a few drops of a solution of calcium chloride. Evidently, therefore, in the precip-

itation of the fibrinogen some prothrombin was carried down with it, and it was interesting to inquire whether it was all thus precipitated with the fibrinogen. To determine this point the original plasma, after the removal of the fibrinogen, was dialyzed against 0.9 per cent solution of sodium chloride to reduce the amount of sodium chloride in it, and was then tested for the presence of prothrombin. It was found that this dialyzed liquid added to a solution of pure fibrinogen gave no clot, but that if a little calcium chloride was also added coagulation occurred in a short time. It is scarcely necessary to add that calcium chloride added to a pure solution of fibrinogen has no effect. From these two experiments it would seem that the prothrombin, as we should expect, is carried down in part only by the precipitated fibrinogen. In the usual method of purifying the fibrinogen by several (3) successive precipitations the prothrombin may be gotten rid of completely, but it is obvious that all preparations of fibrinogen used in experiments upon coagulation should be examined in regard to this point by testing their coagulability after the addition of calcium chloride.

Experiments upon the production of thrombin from other proteins.—It has long been stated that thrombin (fibrin ferment) may be produced from other proteins especially during the process of putrefaction. Some of these statements rest undoubtedly upon a confusion between thrombin action proper and zymoplastic or thromboplastic action. Many substances will facilitate the reaction between fibrinogen and thrombin or fibrinogen and prothrombin, but no substance except thrombin or some derivative compound is capable of converting a pure solution of fibrinogen to fibrin. On such solutions zymoplastic substances are entirely without action. But the conclusion arrived at in this investigation, namely, that the thrombin is a protein body of probably simpler structure than the usual animal proteins, made it probable that it might be produced from such proteins by processes of hydrolysis capable of being controlled. The experiments on this line have not been wholly successful and will be described very briefly. Attempts were made to prepare the thrombin from fibrin by acid hydrolysis, pancreatic hydrolysis, and by putrefaction. The product in each case was treated to isolate basic proteins; that is to say, it was precipitated while hot with a hot acid solution of phosphotungstic acid, the precipitate was filtered off, washed with 5 per cent

sulphuric acid containing some phosphotungstic acid, and treated with hot solution of barium hydrate. After filtration the excess of barium was removed by careful additions of dilute sulphuric acid and the neutral filtrate was evaporated to dryness at a low temperature and its action was tested upon pure solutions of fibrinogen. These solutions caused always a precipitation of the fibrinogen but not a coagulation, except in the case of the putrefied fibrin. This preparation treated as outlined above gave solutions which in some cases when added to a fibrinogen solution threw down at once a gelatinous precipitate resembling a clot. It would seem that the thrombin present in quantity in the putrefied solution of fibrin had been carried through the process given above without wholly losing its power of throwing down fibrinogen in the form of a gelatinous precipitate. The process differed from that of normal coagulation in the immediate production of a precipitate. Insufficient quantities allowed to act for a long time gave no effect, while further addition threw down a gelatinous precipitate at once. A somewhat more promising result was obtained from the action of bacteria. Through the kindness of Dr. W. W. Ford cultures were made in bouillon and Dunham's solution with the bacillus coli communis, proteus vulgaris, and bacillus of Metchnikoff. These cultures were passed through a Berkefeld filter and were then tested upon a solution of pure fibrinogen with their normal alkaline reaction and after neutralization with dilute acetic acid. None of the specimens had clotted after five hours, and it was then necessary to leave them unobserved for thirty hours. At the end of that time the culture of the colon bacillus in broth, both neutral and alkaline, had caused a clot in the fibrinogen, while the culture of the same bacillus in Dunham's solution and the cultures of the other bacteria were without action. It has not been possible as yet to follow this suggestion further.

Intravenous injection of thrombin. — It would seem probable that the very powerful specimens of pure thrombin prepared by the chloroform method might cause a distinct effect if brought directly into the circulation of a living animal. A single experiment of this kind gave, however, entirely negative results. A small dog was used weighing 3750 gm. It was anæsthetized with morphia and ether, and, while connected with a kymographion for the registration of blood pressure, solutions of thrombin were injected into the femoral vein.

Three injections were made, — 16 mgm. thrombin at 10.04 A. M.; 16 mgm. at 10.10 A. M., and 32 mgm. at 10.20 A. M. None of these injections produced any effect upon the pulse rate or blood pressure. Dr. Duke kindly determined the coagulation time of the blood during the experiment from drops of blood drawn from the ear. The normal coagulation time was from five to seven minutes. After the second injection there was a slight but temporary lengthening of this period to ten minutes. The blood plates examined in Wright's solution showed no indications of agglutinating. The results of the injection were therefore entirely negative, and indicate that the body can normally neutralize and render harmless quite large doses of thrombin introduced suddenly into the circulation.

SUMMARY OF RESULTS.

1. A new method is described for preparing thrombin free from admixed protein. The method consists in treating washed fibrin with dilute salt solution (8 per cent sodium chloride) to dissolve the thrombin and then precipitating the coagulable protein by repeated treatments with chloroform.

2. Pure thrombin gives reactions which indicate that it is a simple protein. It does not contain phosphorus or sulphur, gives positive reactions with the biuret, millon, and especially the tryptophan tests, is not coagulated by boiling in neutral solutions, is very readily soluble in pure water, is completely precipitated unchanged by half saturation with ammonium sulphate. In weakly acid solutions (acetic acid) it gives on heating a solution which shows a turbidity on subsequent cooling.

3. Thrombin when allowed to stand in solution for long periods, protected from putrefaction by the addition of chloroform, gradually undergoes an alteration and eventually loses its power of coagulating fibrinogen. This change is hastened by a high temperature. Solutions of thrombin allowed to undergo putrefaction show first an increased power of coagulating fibrinogen followed after a long period by a complete loss of coagulating effect.

4. Solutions of thrombin containing some sodium chloride may be heated to the boiling temperature without losing completely their coagulating action on fibrinogen. Dialyzed solutions of thrombin

are destroyed more completely and rapidly by exposure to high temperatures.

5. Thrombin when dried at low temperatures and protected from moisture in a desiccator may be kept indefinitely.

6. Thrombin probably does not act upon fibrinogen after the manner of an enzyme. Increasing amounts of thrombin give increasing amounts of fibrin, although in decreasing proportion. The weight of fibrin produced by a given submaximal amount of thrombin is not affected by the time during which the thrombin is allowed to act. When minimal amounts of thrombin are used, one part of thrombin can convert at least 215 times its weight of fibrinogen to fibrin.

7. In the non-coagulable peptone plasma of the dog there is contained an antistubstance (antithrombin) which prevents the action of thrombin on fibrinogen. This antistubstance is not destroyed by a temperature of 60° C., but is destroyed at 75° to 80° C. Dilution with water causes spontaneous coagulation in peptone plasma; dilution with saline solution (0.9 per cent sodium chloride) has no such effect.

8. Prothrombin may be converted to thrombin in solutions free from calcium salts.

9. Large amounts of pure thrombin may be injected into the circulation of the living animal without any noticeable effect.

THE UNIVERSITY PRESS, CAMBRIDGE, U. S. A.